

Amendments to the Claims:

This listing of claims will replace all prior versions, and listings, of claims in the application:

Listing of Claims:

1. (Canceled)

2. (Currently Amended) The method according to any one of claims 11-19 wherein the pufferfish type I collagen extract is administered ~~Use of a pufferfish type I collagen extract as effective component in the manufacture of a medicament and health care food according to claim 1, characterized in that said medicament and health care food are in the form of an oral preparation.~~

3. (Currently Amended) The method according to any one of claims 11-19 wherein ~~Use of a pufferfish type I collagen extract as effective component in the manufacture of a medicament and health care food according to claim 1, characterized in that said~~ the pufferfish type I collagen extract is prepared from pufferfish skin and/or pufferfish bone including fins.

4. (Original) A process for the production of a pufferfish type I collagen extract from pufferfish skin and/or pufferfish bone including fins, comprising the following steps:

1) pre-treating raw materials:

a) pre-treating natural pufferfish skin and bone raw material to remove toxin: treating the raw material in an acid solution or alkaline solution at 0 to 50°C for 4 to 48 hours, sufficiently washing with water, and repeating this step for 4 to 6 times; wherein when an alkaline solution is used for the removal of toxin, the preferred conditions are: normal pressure, a final alkaline solution concentration of 0.01

to 0.1 mol/L, 20-30°C, detoxifying for 8 to 24 hours, and repeating for 4 to 5 times; and when an acid solution is used for the removal of toxin, the preferred conditions are: normal pressure, a final acid solution concentration of 0.1 to 0.2 mol/L, 0-20°C, detoxifying for 6 to 24 hours, and repeating for 4 to 5 times;

b) washing clearly the skin and bone of the pufferfish artificially bred in fresh water or washing the detoxified skin and bone of natural pufferfish with water, and storing at -20°C or below for standby if it is not used immediately;

2) extracting according to one of the following three methods:

a) adding water or acid solution to the pre-treated raw material of pufferfish skin and bone in any proportion, extracting at a temperature of from 0 to 125°C, a pressure of from normal pressure to 3 atms for 60 minutes to 100 hours, filtering to obtain the liquid portion, repeating for 0 to 6 times, combining the filtrates, adding water to the residue or combining the filtrates with the residue, homogenizing to obtain a homogenate, standing the homogenate obtained by the acid solution as extracting solvent at 20°C or below for 12 to 48 hours; the homogenate obtained by water as extracting solvent will be directly used in the next step; wherein when the acid solution is used for the extraction, the preferred conditions are: normal pressure, 0 to 10°C, a final acid solution reaction concentration of 0.1 to 0.5mol/L, extracting for 48 to 72 hours, repeating for 2 to 4 times, homogenizing; and normal pressure, 40 to 80°C, a final acid solution reaction concentration of 0.01 to 0.2 mol/L, extracting for 4 to 8 hours, repeating for 3 to 5 times, and homogenizing; wherein when water is used for the extraction, the preferred conditions are: normal pressure, 90 to 100°C, extracting for 3 to 8 hours, repeating for 1 to 3 times, and homogenizing;

b) adding water or acid solution to the pre-treated pufferfish bone raw material, extracting at a temperature of from 0 to 125°C and a pressure of from normal pressure to 3 atms for 60 minutes to 100 hours, filtering to

obtain the liquid portion, repeating for 0 to 6 times, combining filtrates, discarding residue, concentrating the filtrates to 100% to 10% of the original volume, adding an amount of pufferfish skin raw material, extracting at a temperature of from 0 to 125°C and a pressure of from normal pressure to 3 atms for 60 minutes to 100 hours, filtering to obtain the liquid portion, adding water or the same acid solution for extraction, and repeating for 0 to 6 times, combining filtrates, combining the filtrates and the residue, homogenizing to obtain a homogenate, standing the homogenate obtained by the acid solution as extracting solvent at 20°C or below for 12 to 48 hours; the homogenate obtained by water as extracting solvent will be directly used in the next step; wherein when the acid solution is used for the extraction, the preferred conditions are: normal pressure, 0 to 10°C, a final acid solution reaction concentration of 0.1 to 0.5mol/L, extracting for 48 to 72 hours, repeating for 2 to 4 times, homogenizing; and normal pressure, 40 to 80°C, a final acid solution reaction concentration of 0.01 to 0.2 mol/L, extracting for 4 to 8 hours, repeating for 3 to 5 times, and homogenizing; wherein when water is used for the extraction, the preferred conditions are: normal pressure, 90 to 100°C, extracting for 3 to 8 hours, repeating for 1 to 3 times, and homogenizing;

c) obtaining the pufferfish type I collagen extract of the present invention by the conventional methods or modified methods for extracting type I collagen and gelatin in the prior art;

3) filtrating and concentrating:

centrifuging or filtering the homogenate to remove residue, optionally concentrating the filtrate to 100% to 10% of the original volume to obtain a concentrated pufferfish type I collagen extract; wherein when the extraction is carried out by using acid solution at low temperature, the preferred method for removing residue is high speed centrifugation at a low temperature; while when the extraction is carried out by using water or the acid solution at high temperature, the preferred method for removing residue is filtration; when the extraction is carried out by using acid solution at low temperature, the preferred concentration method is the concentration by

ultrafiltration using a ultrafiltration membrane with a pore diameter of 100 to 200 Kda; when the extraction is carried out by using water or acid solution at high temperature, the preferred concentration method is vacuum concentration; the preferred simple production method comprises: after the aforementioned extract is centrifuged or filtrated to remove residue, it is directly subjected to (ultrafiltration) concentration and (freeze, spray) drying to obtain the pufferfish type I collagen extract;

4) optionally, drying and pulverizing:

drying the extract or the concentrated extract (spray-drying, freeze-drying, or microwave-drying, drying by baking, drying in the shade, preferably freeze-drying or spray-drying), pulverizing to obtain a pufferfish type I collagen extract, a light-yellow or white powdery product capable of passing through a 80 mesh sieve; wherein the acid solution is an organic acid or inorganic acid; the alkaline solution is an inorganic alkaline solution; the final concentration in the extraction step is 0.001 to 1.0mol/L; the final concentration in the detoxification step is 0.01 to 0.5mol/L; the examples of the used acid are: formic acid, acetic acid, propionic acid, malonic acid, butyric acid, succinic acid, malic acid, citric acid, tartaric acid, lactic acid, phosphoric acid, hydrochloric acid, sulfuric acid, nitric acid; the examples of the used alkali are: sodium hydroxide, potassium hydroxide, calcium hydroxide (lime water), sodium carbonate; the examples of the used enzyme are: trypsin, pancreatin, pepsin, papain, chymotrypsin, bromelain, dispase, pronase, fibrin, gelatinase, type II collagenase, type III collagenase, proteinase K, and various proteinases from other animals, plants and microorganisms.

5. (Original) A process according to claim 4, characterized in that a controllable partial hydrolysis step is performed according to one of the following two methods is performed after the filtration and concentration steps:

1) hydrolyzing by using a proteinase under conditions of: a proteinase concentration of 1 to 100mg/100g wet weight tissue, preferably 10-50mg/100g wet weight tissue in the reaction system, stirring, a temperature of 20-65°C, preferably

30-37°C, a time of 3-100 hours, preferably 3-48 hours, heating to 100°C for 5-10 minutes to inactivate the enzyme after the end of enzymolysis;

2) hydrolyzing by using an organic acid and/or an inorganic acid under conditions of: an acid concentration of 0.001-1.0mol/L, preferably 0.05-0.50mol/L in the reaction system, stirring, a temperature of 0-100°C, preferably 25-75°C, a time of 60 minutes to 72 hours, preferably 3 to 24 hours, neutralizing or removing acid under vacuum;

optionally, concentrating the hydrolysis solution to a volume of 100% to 10% of the original volume, and drying the concentrated hydrolysis solution to obtain a pufferfish type I collagen extract, or precipitating and drying to obtain a pufferfish type I collagen extract; wherein the used acid and enzyme are those defined in claim 4; the preferred enzyme is type III collagenase, trypsin, pepsin; and the preferred acid is acetic acid and hydrochloric acid.

6. (Original) A process according to claim 4, characterized in that a sedimentation step is performed according to one of the following two methods after the concentration step:

1) adding to the concentrated extract a cold acetone having a volume of 8 to 15 times, preferably 10 to 12 times the volume of the concentrated extract, sedimentating at 10°C or below for 24 to 48 hours, centrifuging or filtering to obtain a precipitate, volatilizing the residual organic solvent from the precipitate, and drying to obtain the pufferfish type I collagen extract;

2) adding to the concentrated extract a cold ethanol until the final ethanol concentration reaches 55-90%, preferably 75-90%, sedimentating at 10°C or below for 24-48 hours, centrifuging or filtering to obtain a precipitate, volatilizing the residual organic solvent from the precipitate, optionally drying to obtain the pufferfish type I collagen extract.

7. (Original) A process according to claim 6, characterized in that the obtained precipitate is repetitively extracted by a neutral buffer (pH 7.5) of 1.0 to 2.2mol/L NaCl or directly by 1.0 to 2.2mol/L NaCl solution, the extract solution is desalted, and optionally dried to obtain a pufferfish type I collagen extract with a relatively high purity.

8. (Currently Amended) ~~A—The process according to any one of claims 4–to–7, wherein the concentrated solution obtained in step 3) of claim 4, the hydrolysis solution of claim 5, the solution of redissolving the precipitate of claim 6, and the extract solution of claim 7 are~~ is neutralized, filtered and desalted, and then ~~are~~ is purified by DEAE- and/or CM-ion exchange chromatography ~~method~~ to remove other protein impurities, and the eluent of the ion exchange is desalted and dried to obtain a pufferfish type I collagen extract with a high purity.

9. (Currently Amended) A pufferfish type I collagen extract prepared by a process according to any one of claims ~~4–to–8~~ and 20–22.

10. (Currently Amended) A pufferfish type I collagen extract ~~according to prepared by a process according to any one of claims 4–8 and 20–22~~ claim 9, characterized in that the main chemical components and pharmacologically active components of said pufferfish type I collagen extract are nature pufferfish type I collagen or denatured pufferfish type I collagen and partial hydrolytes thereof, and the pufferfish type I collagen extract has the following features:

a) the pufferfish type I collagen extract is prepared by using pufferfish skin and/or bone (fins) as raw materials, comprises pufferfish type I collagen or denatured type I collagen and partial hydrolytes thereof as main chemical components and pharmacologically active components, and has physical/chemical properties of typical (fish) type I collagen;

b) the content of pufferfish type I collagen or denatured pufferfish type I collagen and partial hydrolytes thereof in the pufferfish type I collagen extract is greater than 50%, and the total protein content is greater than 70%;

c) the molecular weight of pufferfish type I collagen protein trimer [$\alpha 1(I)$]₂ $\alpha 2(I)$ is from 300 to 420KDa, and the molecular weight of the denatured pufferfish type I collagen protein (comprising $\alpha 1(I)$ monomer, $\alpha 2(I)$ monomer, $\alpha 1(I)$ ₂ dimer and $\alpha 1(I)\alpha 2(I)$ dimer) and partial hydrolytes thereof is from 60 to 300 KDa; the isoelectric points of the two subunits of the pufferfish type I collagen protein separately are $\alpha 1(I)$:4.85±0.5 and $\alpha 2(I)$:6.71±0.5 (see Fig. 1) according to isoelectric focusing polyacrylamide gel electrophoresis method, while the isoelectric points of the two subunits of pufferfish type I collagen protein may vary according to the species of pufferfish;

d) the results of the ultraviolet absorption scanning by using Perkin Elmer Lambda 2 UV-Vis Spectrometer show that the maximum wavelength of ultraviolet absorption of 0.3mg/ml pufferfish type I collagen extract solution obtained by using 0.2mol/L acetic acid as solvent is 226±3nm; while the maximum wavelength of ultraviolet absorption of 0.1mg/ml pufferfish type I collagen extract solution obtained by using 0.1mol/L hydrochloric acid as solvent is 203±3nm; and further, there is no absorption peak in the range from 260 to 280nm and the absorption value in said wavelength range is relatively low (see Fig. 3);

e) According to the measurements of Kivirikko method and automatic amino acid analyzer, the pufferfish type I collagen extract has a weight percentage content of hydroxyproline of greater than 4.5% that is similar to that of other fish collagen, the weight percentage content of hydroxyproline of fish collagen is usually lower than 10%, which is significantly lower than the content of hydroxyproline (14%) in the collagen of terrestrial animal; the amino acid components of the pufferfish type I collagen extract obtained by a process according to any one of claims 4 to 8 are shown in Tables 1, 2, 3 and 5; the pufferfish type I collagen extract is a glycoprotein and has a protein-bound carbohydrate content of 0.5 to 1.9%; and it is understandable that the data

difference is caused by different raw materials and different extraction conditions, but they are measured based on pufferfish type I collagen as main chemical component and pharmacologically active component;

f) the pufferfish type I collagen extract is soluble in water and dilute acid solution, wherein the water solution is thermostable and can maintain its pharmacological activity after being heated at 95 to 100°C for several hours; the dilute weak acid solution of pufferfish type I collagen extract (less than 0.5mol/L) maintains a substantially stable pharmaceutical activity after being placed at a temperature from -20°C to room temperature for a long period; however, the pufferfish type I collagen extract is very sensitive for alkali and may fully lose its pharmaceutical activity in a weak alkali solution, even it is merely placed at room temperature for several hours; the pufferfish type I collagen extract is not sensitive for type III collagenase, and is sensitive for type I collagenase; after the pufferfish type I collagen extract is hydrolyzed by a type I collagenase, its pharmacological and biological activity decreases quickly.

11. (New) A method of preventing or treating a gastrointestinal disease comprising administering to a human with a gastrointestinal disease a pufferfish type I collagen extract.

12. (New) The method of claim 11, wherein the gastrointestinal disease is selected from the group consisting of gastric ulcer, alcohol-induced gastric ulcer, drug-induced gastric ulcer, gastrorrhagia, alcohol-induced gastric mucosa injury, drug-induced gastric mucosa injury, stress gastric ulcer, acute gastritis, chronic gastritis, superficial erosive gastritis, erosive gastritis, gastrospasm, gastralgia, bile reflux gastric ulcer, duodenal ulcer, irritable bowel syndrome, colonitis, gastrointestinal dysfunction, gastric kinetics disorder, indigestion, malabsorption and body weight loss, abdominal distension and diarrhea caused thereby.

13. (New) A method of preventing or treating liver cell damage comprising administering to a human with liver cell damage a pufferfish type I collagen extract.

14. (New) The method of claim 13 wherein the liver cell damage comprises damage selected from the group consisting of alcoholic liver damage, hepatic fibrosis, hepatic cirrhosis, alcohol-induced hepatic cirrhosis, alcohol-induced hepatic fibrosis, drug-induced liver damage, drug-induced hepatic fibrosis, and drug-induced hepatic cirrhosis.

15. (New) A method of preventing or treating a collagen proliferative disease comprising administering to a human with a collagen proliferative disease a pufferfish type I collagen extract, wherein the proliferative disease is kidney fibrosis or myocardial fibrosis.

16. (New) A method of preventing or treating an immune disease, comprising administering to a human with an immune disease a pufferfish type I collagen extract, wherein the immune disease is selected from the group consisting of immune dysfunction, leucopenia, rheumatoid arthritis, rheumatic arthritis, lupus erythematosus.

17. (New) A method of preventing the development of or treating cancer, comprising administering to a cancer patient a pufferfish type I collagen extract, wherein the cancer is selected from the group consisting of malignant gastric tumors, gastric cancer, liver cancer, colon cancer, rectal cancer, solid malignant tumors.

18. (New) A method preventing or treating the development of metastasis of a cancer, comprising administering to a cancer patient a pufferfish type I collagen extract, wherein the cancer is selected from the group consisting of malignant

gastric tumors, gastric cancer, liver cancer, colon cancer, rectal cancer, solid malignant tumors.

19. (New) A method of preventing or treating angiogenesis-associated diseases, comprising administering to a human with an angiogenesis-associated disease a pufferfish type I collagen extract.

20. (New) The process according to claim 5, wherein the hydrolysis solution is neutralized, filtered and desalted, and then is purified by DEAE- and/or CM-ion exchange chromatography to remove other protein impurities, and the eluent of the ion exchange is desalted and dried to obtain a pufferfish type I collagen extract with a high purity.

21. (New) The process according to claim 6, wherein the solution of redissolving the precipitate is neutralized, filtered and desalted, and then is purified by DEAE- and/or CM-ion exchange chromatography to remove other protein impurities, and the eluent of the ion exchange is desalted and dried to obtain a pufferfish type I collagen extract with a high purity.

22. (New) The process according to claim 7, wherein the extract solution of claim 7 is neutralized, filtered and desalted, and then is purified by DEAE- and/or CM-ion exchange chromatography to remove other protein impurities, and the eluent of the ion exchange is desalted and dried to obtain a pufferfish type I collagen extract with a high purity.